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Molecular Mechanisms for Organ-specific Colon Carcinoma Metastasis

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The mechanistic basis of a metastatic cell's ability to proliferate in the parenchyma of certain organs and develop organ-specific metastases remains largely unknown. Signals from paracrine and/or autocrine pathways may regulate tumour cell proliferation, with the eventual outcome dependent on the net balance of stimulatory and inhibitory factors. Recent data demonstrate that organ microenvironments can modulate gene expression of tumour cells, including regulation of growth at the organ-specific metastatic site. Analyses of highly metastatic human colon carcinoma (hCC) cells selected in nude mice as well as *in situ* mRNA hybridisation analyses of archival colon carcinoma specimens correlated high levels of epidermal growth factor receptor with the malignant hCC cell's ability to grow in the liver parenchyma. These same metastatic cells can also respond to specific mitogens produced by tissue undergoing repair, demonstrating that physiological signals can be utilised by neoplastic cells. This article will address experimental evidence supporting the premise that organ-derived, paracrine growth factors regulate the growth of malignant cells that express the appropriate receptors.

Key words: organ-specific metastasis, epidermal growth factor receptor, growth factors, liver regeneration, host–tumour interactions

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INTRODUCTION

CANCER OF the colon and rectum is the second most prevalent cause of cancer deaths in men and the third most common in women. The majority of patients with colon cancer present with either Dukes' stage B or C disease, with overall survival for patients undergoing surgical excision being 60–85% and 40–60%, respectively. Approximately 55% of patients who have undergone surgery for the removal of their primary colon tumour will recur within 5 years. Of these patients, half will recur regionally, and up to 80% will have distant metastases, of which the liver is the most common site [1]. Surgical treatment of these metastases is effective as a curative therapy only in a small number of cases, and chemotherapy and radiation therapy are largely palliative [2]. Therefore, a primary goal in our research is understanding the molecular mechanisms mediating not only the development of primary colorectal cancers, but the process of colon carcinoma metastasis.

Metastasis is defined as the spread of cells from a primary neoplasm to distant secondary sites and proliferation at these sites. This highly selective process consists of a series of linked, sequential steps favouring the survival of a subpopulation of metastatic cells that pre-exist within the primary tumour mass [3]. These steps include the detachment of cells from the primary tumour, invasion of the surrounding tissue, penetration into the circulation (bloodstream or lymphatic system), implantation into the capillary beds of target organs, extravasation and invasion into the target tissue, formation of a vascular network, and finally proliferation at this secondary site of implantation [3, 4]. For production of clinically relevant metastases, each of these steps must be completed. Failure to complete even one step in this process (e.g. the inability to invade host stroma, a high degree of antigenicity, inability to grow in a distant organ's parenchyma) eliminates the cells. The successful metastatic cell must, therefore, exhibit a complex phenotype that recent experimental evidence suggests is regulated by transient or permanent changes in different genes at the DNA and mRNA level(s) [4].

Numerous examples exist in which malignant tumours metastasise to specific organs. As Paget proposed in 1889 [5], the organ microenvironment (the "soil") can influence the implantation, invasion, survival, and growth of particular tumour cells (the "seeds"). This hypothesis, supported experimentally [6] and clinically [7], explains metastatic colonisation patterns that cannot be explained solely by mechanical lodgment theories and anatomical considerations [8]. Hence, successful metastasis depends, in part, on the interaction of favoured tumour cells with a compatible milieu provided by a particular organ environment [3, 9]. The precise mechanisms mediating this interaction remain largely unknown, although recent experimental evidence using different model systems, including human colon carcinoma, suggests that paracrine stimulation of tumour cells by organ-derived growth factors (GFs) is one potential mechanism by which target organ preference of disseminated cancer cells is determined [3, 9]. Therefore, a modern interpretation of Paget's hypothesis is that the microenvironment of each organ is capable of influencing the phenotype of particular tumour cells. Recent reports confirming this interpretation have demonstrated that the organ microenvironment can profoundly influence the bio-

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logical behaviour of metastatic tumour cells, including resistance to chemotherapy [10], the production of degradative enzymes [11], angiogenesis [12], melanogenesis [13], induction of terminal differentiation [14], and growth at the organ-specific site [15, 16]. This article will review these data, with emphasis on the known molecular mechanism(s) responsible for the proliferation of metastatic human colon carcinoma (hCC) cells at the liver-specific secondary site, in terms of differentially expressed local paracrine GFs or organ-repair factors which affect the growth of the malignant cell that expresses the appropriate receptor(s).

LIVER-SPECIFIC METASTASIS OF HUMAN COLON CARCINOMA XENOGRAFTS IN ATHYMIC NUDE MICE

The cellular and molecular mechanisms which regulate the metastasis of tumour cells to specific organs are diverse and both tumour and organ-specific [3, 6, 9]. Evidence from metastatic models of hCC have shown that an appropriate model for human cancer metastasis must use metastatic cells, and they must grow in a relevant organ environment (for review see [17]). A Dukes' stage B2 primary hCC was found to contain heterogeneous populations of cells with different metastatic properties only after the cells were implanted into the anatomically correct sites in athymic nude mice (orthotopic implantation) [17]. Implantation of these hCC cells into the spleen or caecum of nude mice produced exclusively hepatic and lymph node metastases; whereas, implantation of these same cells at ectopic sites (e.g. subcutaneous or intramuscular) resulted in slow growth of primary tumours and rare formation of metastases [17]. Orthotopic implantation of human neoplasms is thus necessary for expression of the metastatic phenotype, and if a human tumour is biologically heterogeneous, some of its cells may possess a growth advantage, depending on whether it is transplanted to the skin, the caecum, the liver, or the kidney of nude mice [9]. These results demonstrate the significance of anatomical compatibility of tumour cells ("seed") with the organ environment ("soil").

A reproducible assay of hepatic metastasis was utilised to distinguish the malignant potential of different stage hCCs whereby tumour cells from hCC surgical specimens were inoculated into the spleens of nude mice, and their growth analysed in the liver parenchyma [3, 17]. The growth of hCC in the liver directly correlated with the metastatic potential of the cells, i.e., tumour cells isolated from surgical specimens of primary hCC, classified as either modified Dukes' stage D or liver metastases, produced significantly more colonies in the liver of nude mice than tumour cells isolated from a Dukes' stage B tumour [17]. Radioactive distribution analyses of both Dukes' stage B and D hCC cells demonstrated that, shortly after intrasplenic injection, similar numbers of tumour cells reached the liver microvasculature; thus, the presence of viable tumour cells in an organ does not predict if the cells will proliferate to produce metastases. These results are consistent with experiments using radiolabelled tumour cells (rodent or human) from different model systems which showed that tumour cells reached the microvasculature of many organs, but growth *per se* occurred in only specific organs [3, 6, 9]. These experiments emphasise that the sites of metastasis are determined not only by the characteristics of the neoplastic cells, but also by the microenvironment of the host tissue. Therefore, the production of hCC metastases in the livers of nude mice was determined in part by the ability of the hCC to proliferate in the liver parenchyma rather than by the ability of the cells to reach the liver [15, 17].

To select and isolate metastatic subpopulations of hCC cells with increasing growth potential in the liver parenchyma from heterogeneous primary hCCs, cells were derived from a surgical specimen of a Dukes' stage B2 primary hCC. These hCC cells were established in culture (KM12C) or injected into the subcutis, caecum, and spleen of nude mice [17]. Progressively growing tumours were then isolated and established in culture. Implantation of these four culture adapted lines into the caecum or spleen of nude mice produced few metastatic foci in the liver. hCC cells from these few liver metastases were expanded into culture and re-injected into the spleen of nude mice to provide a source for further cycles of selection. Importantly, with each successive *in vivo* selection cycle, the metastatic ability of the isolated and propagated cells increased. Four cycles of intrasplenic selection yielded cell lines (KM12L4) with a very high metastatic efficiency in nude mice. In analogous studies of Dukes' stage D primary hCC, highly metastatic cell lines were isolated, but successive selection cycles for growth in the liver only slightly increased their metastatic properties [17]. These results indicate that highly metastatic subpopulations of tumour cells can be selected from early stage hCC, and that orthotopic implantation of hCC cells in nude mice is a valid model for evaluating metastatic potential.

LIVER-SPECIFIC REPAIR PROCESSES AND GROWTH FACTORS

A mechanism for the site-specific growth of metastases involves interactions between receptive tumour cells and the organ environment in terms of responses to local GFs. Evidence supporting an association between organ-derived GFs and receptive metastatic cells was obtained, in part, from experiments on the effects of organ-conditioned medium on the growth of particular neoplastic cells. The presence of stimulatory or inhibitory factors in a particular tissue correlated with the organ-specific pattern of metastasis (for reviews see [6, 9]). Metastatic cells may, therefore, proliferate in secondary organs which produce compatible GFs; that is, GFs similar or identical to those involved in the cellular regulation of the normal tissue from which the primary tumour originated [9, 15]. For example, hCC tumours utilise and respond to specific GFs which regulate normal colonic epithelium [9]. Some of these identical factors also regulate homeostasis and tissue renewal and repair in the liver (i.e. transforming growth factor-alpha (TGF-α) and hepatocyte growth factor (HGF); see below). Therefore, do these same factors and receptors participate in the regulation of hCC growth at the metastatic liver-specific site? Recent data suggest that this may be one mechanism. For example, transplantation experiments with a hCC cell line, KM12C, were performed in nude mice that had been subjected to either hepatectomy (60%), where the liver undergoes rapid cell division termed regeneration, or trauma control abdominal surgery [18]. hCC cells implanted subcutaneously demonstrated accelerated growth in partially hepatectomised mice but not in control mice, indicating that liver regeneration in the nude mouse stimulated growth of the KM12C colon carcinoma cells. van Dale and Galand, using intraportal inoculated rat colon adenocarcinoma cells, showed a dramatic increase in the incidence and growth of tumour cell colonies in the liver of partial hepatectomised rats as compared to sham-operated controls [19]. These findings suggest that signals required for repair of damaged tissues can be recognised by neoplastic cells. Consistent with these obser-

vations is the appearance during liver regeneration of factors in the peripheral blood that stimulate DNA synthesis in grafted hepatic parenchyma concomitant with the DNA synthesis of the liver *in situ* [20]. Thus, tumour cells that either originate from or have affinities for growth in a particular organ can respond to organ-derived signals.

Liver regeneration which follows partial hepatectomy involves quantitative changes in hepatocyte gene expression [20]. TGF-α mRNA was shown to increase approximately 2-fold in hepatocytes during the first 8–24 h following partial hepatectomy, coinciding with an increase in EGF-R mRNA and a down-regulation of these receptor proteins, as well as a loss of EGF-dependent protein kinase activity [21]. These results, in addition to data of TGF-α stimulation of hepatocytes *in vitro*, strongly suggest that TGF-α may be a physiological regulator of liver regeneration by means of an autocrine mechanism [21]. TGF-α production by hepatocytes might also have a paracrine role, stimulating proliferation of adjacent nonparenchymal cells [20, 21]. HGF is another mitogen involved in liver regeneration [20]. In experimental animals treated with hepatotoxins or with other kinds of liver injuries, such as ischaemia, physical crushing, or 60% partial hepatectomy, the levels of HGF protein and HGF activity in the plasma increase very rapidly during the early phase of regeneration: whereas TGF-α levels increase later, implying that HGF may be involved in triggering the hepatocytes to enter the cell cycle. The amount of HGF transcribed also rises not only in the liver of injured animals, but also in the lung, spleen, and kidney, suggesting endocrine as well as paracrine functions for HGF in liver regeneration [20]. Direct evidence that HGF is involved in the process of liver regeneration comes from studies in which purified HGF was injected into mice, after which the number of hepatocytes entering DNA synthesis was monitored. These data demonstrated that HGF enhanced liver regeneration in mice which had been subjected to hepatectomy or carbon tetrachloride treatment [20]. These data demonstrate that both TGF-α and HGF are important mediators of liver regeneration and hepatocyte proliferation. Therefore, when normal tissues are disturbed or damaged, growth factors are released to stimulate normal organ tissue repair, and indirectly, may stimulate the proliferation of receptive malignant tumour cells [6, 9].

EXPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGF-R) PREDICTS THE METASTATIC POTENTIAL OF hCC

TGF-α exerts its effect through interaction with the EGF-R. This plasma membrane glycoprotein contains, within its cytoplasmic domain, a tyrosine kinase activity. The binding of TGF-α to the EGF-R stimulates a series of rapid responses, including phosphorylation of tyrosine residues within the EGF-R itself and on other cellular proteins, hydrolysis of phosphatidyl inositol, release of Ca^{2+} from intracellular stores, elevation of cytoplasmic pH, and morphological changes [22]. After 10–12 h in the continuous presence of EGF or TGF-α, cells are committed to synthesise DNA and to divide [22]. EGF-Rs are expressed by many normal and tumour cells [22]. Increased levels of amplification of EGF-R have been found in human tumours and cell lines, including gliomas, lung cancer, bladder cancer and colon carcinoma (for review see [9, 22]). These results suggest the physiological significance of inappropriate expression of the EGF-R tyrosine kinase in normal and abdominal cellular growth control.

Recent data from our laboratory have demonstrated that expression of the EGF-R by hCC cells directly correlates with their ability to produce hepatic metastases [15, 16]. Analysis of the steady-state mRNA expression levels of EGF-R in several hCC cell lines, that were evaluated extensively for their metastatic potential in nude mice (see above), indicated that all highly metastatic cell lines (i.e., those from Dukes' stage D tumours or liver metastases) showed 5–10-fold higher steady-state expression of EGF-R-specific transcripts compared with the low metastatic KM12C hCC cells (derived from a Dukes' stage B_2 primary tumour). Highly metastatic KM12SM cells, derived from a liver metastasis produced by KM12C cells growing in the caecal wall of nude mice, demonstrated a 24-fold increase over the parental steady-state expression of EGF-R transcripts. Similarly, highly metastatic KM12L4 cells, isolated after four successive intrasplenic–liver cyclings, showed levels 14-fold higher than the parental levels of steady-state EGF-R transcripts. Regardless of their metastatic potential, each variant line expressed similar levels of TGF-α mRNA transcripts. These data suggested that the expression level of EGF-R mRNA transcripts directly correlated with the metastatic propensity of the hCC cells in this model system [15].

Western blot analyses demonstrated increased EGF-R-specific protein levels in the highly metastatic KM12L4 and KM12SM cell populations compared with the parental KM12C cells, directly correlating with the observed increases on the transcriptional level. Scatchard analyses and EGF-R-associated protein tyrosine kinase activity assays confirmed these results. TGF-α also stimulated the *in vitro* proliferation of these hCC cells. Parental KM12C cells (low level EGF-R) incubated in medium containing 0.001–1.0 ng/ml TGF-α showed a 20% increase in growth; whereas KM12L4 cells (high level EGF-R) demonstrated 50% growth stimulation in medium containing as little as 0.001 ng/ml TGF-α. These results indicated that TGF-α-mediated growth stimulation of the KM12 HCC variants directly correlated with the number of functional EGF-Rs as shown by abrogation with either EGF-R or TGF-α neutralising antibodies [15].

Single cell analyses of the KM12 HCC variants using immunohistochemistry to evaluate potential heterogeneity in the pattern of EGF-R expression revealed a heterogeneous pattern of cellular staining in the low metastatic KM12C parental population, as illustrated by a few cells with intense staining, a few cells that were negative, and most with intermediate reactivity. In contrast, the highly metastatic KM12L4 and KM12SM variants exhibited a uniformly intense pattern of staining. These results demonstrated that the KM12C cell population was heterogeneous with respect to EGF-R protein expression, and contained few cells with high levels of cell surface EGF-R protein, whereas the highly metastatic KM12L4 and KM12SM variants (isolated from the parental line) exhibited a uniformly high reaction. We, therefore, determined whether the increases in EGF-R mRNA and protein levels were due to an increased copy number of chromosome 7, which contains the gene for EGF-R. We quantified chromosome 7s by fluorescence *in situ* hybridisation and used nonrelevant chromosome 12 as a control. The parental KM12C line was composed of two cell populations: one comprising 70% of the cells, had two copies of chromosomes 7 and 12, and one, comprising 30%, had four copies of each chromosome. In contrast, more than 80% of KM12L4 cells contained four copies of chromosome 7 and three copies of chromosome 12, and a small number of clones contained between five and eight copies of chromosome 7. Analysis of the copy number of chromosomes 7 and 12 simul-

taneously showed that 95% of KM12L4 cells displayed a chromosome 7/12 ratio of >1 compared with only 14% of KM12C cells. The 7/12 ratio was consistently >1 in the minor clones containing between five and eight copies of chromosome 7. These results were further confirmed by quantitation of chromosome 7 versus control chromosomes 4, ruling out loss of chromosome 12 as the cause of the change in the ratio. These data suggested that the observed increase in EGF-R mRNA and protein levels in highly metastatic hCC cells may be due, in part, to an increase in the copy number of chromosome 7.

In the next set of experiments, we isolated the top and bottom 5% EGF-R-expressing cells of the heterogeneous KM12C hCC tumour cell population using fluorescence activated cell sorting (FACS). FACS profile analyses of the sorted cell populations after *in vitro* growth showed that the EGF-R relative fluorescence of the top 5% was approximately twice that of the parental KM12C population, but approximately half that of the *in vivo* selected KM12L4 cells. The bottom 5% of EGF-R-sorted cells and the KM12C parental cells had similar relative fluorescence levels. We tested the potential of the top and bottom 5% EGF-R sorted cell populations to produce experimental liver metastases in nude mice. The top 5% of cells produced a higher incidence and median number of liver metastases compared with either the bottom 5% EGF-R sorted cells or the parental KM12C cells. KM12L4 cells produced the highest number of liver metastases, as expected, correlating with their EGF-R levels. Regardless of EGF-R expression, all cell lines produced the same incidence and size of spleen tumours. These results indicated that expression of EGF-R directly correlates with the ability of HCC cells to grow in the liver parenchyma, and hence, produce hepatic metastases in this model system [15].

IN SITU mRNA HYBRIDISATION FOR EGF-R TRANSCRIPTS IN SURGICAL SPECIMENS OF hCC

Based on our findings that hCC cells isolated from metastases (Dukes' stage D) expressed significantly increased EGF-R transcripts and functional protein receptors as compared to hCC cells with low metastatic potential (Dukes' stage A or B), we analysed archival hCC primary and metastatic surgical specimens for cell-specific EGF-R mRNA expression, using a newly developed rapid colorimetric *in situ* mRNA hybridisation (ISH) technique developed in our laboratory [16]. Analysis of formalin-fixed, paraffin-embedded colon carcinoma surgical specimens for EGF-R mRNA transcripts showed cytoplasmic hybridisation with EGF-R antisense oligonucleotide probes in primary and metastatic colon carcinoma specimens which correlated with immunohistochemistry and northern blot analyses. Furthermore, ISH demonstrated intratumoral heterogeneity in EGF-R gene expression, and identified distinct cells expressing high levels of EGF-R in the tissues [16]. These results indicate that retrospective analyses are feasible using archival human surgical specimens. Analyses of this kind may also determine a clear association of the genetic and phenotypic characteristics/determinants intrinsic to the metastatic hCC cell as well as those components of the surrounding host tissue and their putative roles in the biological progression of colon carcinoma to the malignancy.

CONCLUSION

Recent data from many laboratories have demonstrated that the organ environment can profoundly influence the phenotype of particular tumour cells, thus supporting the hypothesis of Paget that the organ microenvironment (the soil) can influence the implantation, invasion, survival, and growth of particular tumour cells (the seed). The data presented in this article suggest an involvement of the EGF-R in the progression and metastasis of human colon carcinoma, and indicate a potential use of this receptor as a therapeutic target. Possible approaches include using anti-EGF-R antibodies [23, 24], inhibitors of EGF-R tyrosine kinase activity [25, 26], and dominant negative receptor strategies [27, 28].

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